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"Evaluation of methanolic leaf extract of *Olea dioica* Roxb., for antibacterial and anticancer activities"

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Contents

Chapters	Index	Page No.
Chapter I	Introduction and Literature review	1
Chapter II	Materials and Methods	14
Chapter III	Results and Discussion	24
Chapter IV	Summary and Conclusion	37

CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

The world's diverse medicinal plants have been crucial for human health since the beginning of the civilization. They offer an endless supply of active substances for treating various diseases. Medicinal plants are being studied more closely due to their indigenous supply of bioactive chemicals with medicinal properties (Shariff, 2001; Suresh, 2008).

Researchers are increasingly interested in medicinal plants due to their significant role in the pharmaceutical industry. Antimicrobials, which eliminate or impede bacteria's proliferation, have been widely used to treat infections. However, antibiotic resistance is a significant issue due to the indiscriminate use of these drugs. Factors such as bacteria-antibiotic interactions, host characteristics, antibacterial drug usage, and environmental conditions contribute to this resistance (Vaou *et al*., 2021).

Research has focused on finding novel antimicrobial compounds derived from natural sources, such as plants, animals, and microorganisms, which have immense therapeutic potential. Plants, particularly those found in folk medicine, have shown significant antimicrobial activity without causing adverse effects. Around 20% of global plant species have undergone pharmaceutical or biological testing, and a significant proportion of newly introduced antibiotics are derived from natural or semi-synthetic sources. As a result, plants are considered the foundation for modern medical practices aimed at combating infectious diseases (Kadhim *et al.,* 2017).

Plant-derived antimicrobials have been recognized for their therapeutic potential in treating human diseases caused by bacteria, fungus, and viruses. *Staphylococcus aureus*, a Gram-positive bacterium, is associated with post-operative wound infections, severe skin infections, respiratory infections, toxic shock syndrome, endocarditis, osteomyelitis, and foodborne illness. Gram-negative bacteria, such as *Escherichia coli*, are found in the human intestinal tract and are linked to various diseases. *Salmonella typhi*, a Gram-negative bacterium, induces typhoid, a disease spread through contaminated food and water. *Bacillus subtilis*, a Gram-negative bacterium, is found in soil and the gastrointestinal tract. *Pseudomonas aeruginosa*, a multidrug-resistant pathogen, causes serious illnesses in plants, animals, and humans. *Aspergillus niger*, a fungus, is implicated in otomycosis cases, *Aspergillus fumigatus*, *Candida albicans* and *Aspergillus parasiticus* have significant health implications for immune-compromised patients (Akroum *et al.*, 2010).

The antimicrobial properties of phytochemicals are promising, but further research is needed to understand their mechanisms, optimize dosages, and ensure safety. Monitoring

resistance to phytochemicals is crucial. Combining phytochemicals with conventional antibiotics could address multi-drug resistant microorganisms. In India, research on medicinal plants' anticancer activity began in the 1960s and gained momentum in the 1970s. These plants are essential for traditional therapeutic practices due to their affordability, availability, and ancestral knowledge.

Cancer is a lethal disorder characterized by unregulated cell division and the ability to infiltrate other tissues. It is the second-greatest cause of death in developing countries, with a larger burden due to population increase and ageing. In India, breast cancer is the primary cause of fatality among women, with over 5 lakh people dying in 2010 alone. Cancer is classified into various categories based on the organ or cell type it develops in.

Cause of cancer

Cancer is primarily caused by genetic aberrations within cellular genetic material, which are caused by radiation, chemical, and biological agents. Risk factors include lifestyle choices, family history, inheritance, and genetics, as well as environmental exposures like radiation, chemicals, and cancer-causing infections. Age also plays a significant role in cancer incidence. Biological agents, such as viruses, bacteria, and parasites, account for up to 20% of human malignancies globally. Examples include HIV infection, hepatitis B and C virus, cervical cancer, and various types of lymphomas. Epstein-Barr virus, Kaposi's sarcoma herpes virus, human T-cell lymphotropic virus type 1, *Helicobacter pylori*, *Schistosoma hematobium* parasites, and *Opisthorchis viverrin* parasites, all contribute to the development of various types of cancer.

Cancer cells can grow without growth signals, ignore apoptotic signals, and induce angiogenesis, providing oxygen and nutrients. They can evade immune destruction and increase genome alteration and mutation during cell division. They require more energy to multiply, leading to abnormal pathways and reliance on alternative nutrients (Hanahan and Weinberg, 2016).

Cancer is classified based on the type of tissue affected in the human body. Carcinoma the most common cancer, occurs from epithelial tissue, including skin and organs. Melanoma cancer, caused by melanocytes, is caused by pigment-producing cells. Sarcoma, a rare type, occurs in connective tissues and muscle tissue. Leukemia and lymphoma, blood cancers, occur in haematopoietic cells (Bagchi and Preuss, 2004).

Cancer has become a widely recognized term in modern society, with the World Cancer Research Fund International predicting a global incidence of 18.1 million cases in

2020. Breast and lung cancers were the most prevalent malignancies, accounting for 12.5% and 12.2% of newly diagnosed cases, respectively. Colorectal cancer ranked as the third most prevalent form, accounting for around 1.9 million new cases, constituting 10.7% of all newly reported cancer cases. This data highlights the global prevalence of cancer and the need for effective cancer prevention and treatment strategies.

In 2020, lung cancer was the most prevalent form of cancer among men, accounting for 15.4% of new cases. The three most prevalent types were lung, prostate, and colorectal cancers, accounting for 41.9% of all cases. Other prevalent types included stomach and liver cancers. In contrast, breast cancer was the most prevalent malignancy among women, accounting for 25.8% of new cases. Breast, colorectal, and lung cancers were the leading causes, while cervical cancer was the fourth most prevalent.

Cancer treatments include biomarker testing, chemotherapy, hormone therapy, immunotherapy, radiation therapy, stem cell transplantation, surgery, and targeted therapy. The type of cancer, its location, and stage of development determine the type of therapy needed. Most patients receive a combination of therapies, such as surgery and chemotherapy and radiation. However, these treatments can also cause life-threatening side effects like anemia, appetite loss, thrombocytopenia, edema, fertility issues, hair loss, memory problems, neutropenia, lymphedema, urinary problems, and severe pain. As a result, research is growing to develop new drugs from alternative sources. Natural ingredients continue to be a rich source of novel medications, with 80 percent of medications being natural products or naturally inspired compounds (Nurgali *et al*., 2018).

Plants as alternative sources in cancer drug development

Plants have been used as medicine since 2600 BC, with traditional Indian systems like Ayurveda, Siddha, and Unani using medicinal herbs and spices like turmeric for cancer treatment. Plants have emerged as a promising source for developing anticancer drugs due to their diverse bioactive compounds, which have shown anti-cancer activity. Over 3000 plant species have been documented for their therapeutic potential in cancer treatment, with over 60% of the current anticancer drugs tracing back to plants (Fridlender *et al.,* 2015; Varsha *et al.,* 2017). Plant-derived compounds, such as paclitaxel, Vinca alkaloids, camptothecin derivatives, and resveratrol, have shown promising anti-cancer action and are currently being used in cancer therapy. Studies by Iqbal *et al.,* have highlighted the historical relevance of using plants in cancer treatment and the demonstrated efficacy of naturally generated anticancer medications obtained from plants in both preclinical and clinical contexts. This

highlights the potential of plant-derived substances in providing effective and alternative approaches to cancer drug development (Iqbal *et al.,* 2017).

Phytochemicals as anticancer agents

Chemotherapeutic drugs, including topoisomerase inhibitors, alkylating agents, and microtubule-acting agents, have shown promising outcomes in cancer management. However, they have side effects like neutropenia, sensory neuropathy, diarrhoea, and cardiotoxicity. Additionally, they can cause nephrotoxicity, gastrointestinal toxicity, cardiovascular toxicity, pulmonary toxicity, and hematologic toxicity. These drugs also have limitations like high costs, administration intricacies, lack of environmental sustainability, and inherent toxicity (Singh *et al*., 2016).

Cancer is a result of genetic mutations and bacterial or viral contamination, but various targets can delay or inhibit its development through pharmacologic mechanisms. Understanding apoptotic signaling pathways is crucial for complementing current treatments and developing novel therapies. Due to the high cost and side effects of modern treatments, there is a growing interest in chemoprevention as a cost-effective alternative for cancer therapy. Higher plants have the unique ability to synthesize organic compounds known as secondary metabolites, which are considered safer than synthetic compounds. The World Health Organization is interested in screening therapeutic plants and using natural resources in medicine to expand healthcare access. Apoptotic inducers from plants are considered effective cancer preventative medicines due to their low systemic toxicity and resistance to oxidative stress (Uddin and Lee, 2020).

Role of phytochemicals in the prevention of cancer

Phytochemicals, or phytonutrients, are naturally occurring compounds found in plants that have been studied for their potential role in cancer prevention. These compounds have various mechanisms of action, including antioxidant effects, carcinogen inactivation, inhibition of cancer cell proliferation, cell cycle arrest and apoptosis, and immune system control. Phytochemicals found in fruits, vegetables, whole grains, and legumes have been associated with a lower chance of certain cancers (Choudhari *et al.,* 2020).

Phytochemicals, including antioxidants, minerals, and vitamins, have been shown to contribute to cancer chemoprevention through immunomodulatory, antiangiogenic, and cytotoxic effects. They have been found in various sources such as tea, fruits, herbs, vegetables, spices, nuts, seeds, stems, and flowers. The biological effects of flavonoids, alkaloids, diterpenoids, sesquiterpenes, and polyphenols have been extensively documented,

with various antifungal, antimicrobial, antihelmintic, anti-trypanosomal, anti-inflammatory, and antiproliferative activities against various types of cancer (Ranjan *et al.,* 2019).

Approximately 74% of the most significant medications consist of active components originating from plants, with over 3,000 plant species being used for cancer treatment. Preclinical animal models and human epidemiological studies indicate that numerous dietary phytochemicals possess cancer chemo-preventive effects. The most important mechanism by which phytochemicals inhibit carcinogenesis is the scavenging of reactive oxygen/nitrogen species (ROS/RNS) and the initiation of cellular defense mechanisms by antioxidant enzymes. ROS play a pivotal role in intracellular signaling cascades, causing cell function loss, oxidative stress, and apoptosis. Phytochemicals can also induce anti-cancer effects, such as cell cycle arrest by blocking the IKK/NF-B pathway and the signal transducer and activator of transcription (STAT3) pathway(George *et al.,* 2021).

Plant extracts are commonly used in medical practice and have been studied for their potential cytotoxic effects on cancer cells. The MTT assay, which evaluates mitochondrial dehydrogenase activity in cells, is a common method for determining cytotoxicity. This assay measures the capacity of living cells to convert the yellow dye MTT into a purple formazan product.

The MTT assay is a widely used technique for assessing the cytotoxicity of plant extracts on various cancer cell lines. Studies have been conducted on the effects of leaf extracts from *Andrographis paniculata* and *Aspilia africana* on murine cells, medicinal plant extracts from Mazandaran, Iran, and medicinal herbs from Nigerian ethno-medicine on Rhabdomyosarcoma cancer cell lines, and *Cyperus longus* extract, fractions, and essential oil on PC3 and MCF7 cancer cell lines. The lack of comprehensive documentation on the toxicity effects of most medicinal plant extracts can be attributed to the perception that traditional herbs are generally considered safe. The degree of toxicity in extracts is often associated with factors such as concentration, duration of exposure, and physiological parameters(Zhou *et al*., 2014).

Apoptosis Signaling Pathways

Apoptosis, or programmed cell death, is crucial for embryonic development and tissue equilibrium in multicellular organisms. It's controlled and linked to morphological traits like chromatin condensation and cell shrinkage. Dysregulation of apoptosis can lead to diseases like tumor growth and drug resistance (Johnstone *et al*., 2002).

Apoptosis in mammalian cells is triggered by two pathways: the extrinsic pathway, initiated by the activation of death receptors like TNF, and the intrinsic pathway, mediated by the release of mitochondrial molecules like Bcl-2 protein family. The extrinsic pathway is initiated by a ligand binding to death receptors with intracellular death domains, while the intrinsic pathway is activated by various stimuli. Both pathways trigger the activation of initiator caspases, which then trigger effector caspases, a class of cysteine proteases. Aspartate-specific cysteine-dependent proteases are post-translationally regulated, allowing quick activation. These inactive proenzymes are first produced or expressed in cells.

Cell growth and functions are controlled by specific cellular signals in healthy cells. Malignant cells transform when cell death mechanisms are disrupted, such as mutations preventing apoptosis. Inhibiting these altered signals or pathways could be beneficial in combating cancer, as studies have shown.

DNA damage-induced apoptosis

DNA damage-induced apoptosis is a defense mechanism against cancer development by eliminating genetically damaged cells. It involves activating cell cycle checkpoints, stimulating DNA repair, and inducing cell death pathways. Apoptosis is the principal pathway of cell demise following DNA damage and is induced by genomic stress through the death receptor or intrinsic mitochondrial pathway. This process eliminates genetically compromised cells, serving as a cancer defense mechanism.

The tumor suppressor protein p53 is involved in linking DNA damage to apoptosis, a secondary response mechanism that protects against cell proliferation. Research on cell lines with impaired DNA repair mechanisms supports the idea that DNA damage can induce cell death through apoptosis. Different types of DNA lesions can induce apoptosis, which is considered a protective mechanism against cancer (Anichini *et al*., 2006).

DNA damage in mammalian cells temporarily halts the cell cycle for DNA repair, which can trigger cell death mechanisms like apoptosis. This response is crucial for cytotoxic cancer therapies. The inability to repair DNA contributes to cancer's emergence and evolution. DNA repair proteins recognize damage sites and determine whether to repair or push cells towards apoptosis, which is essential for tissue homeostasis and cell elimination.

Cell-Cycle–Mediated Apoptosis in cancer cells

The cell cycle is a normal process involving the synthesis of cellular organelles, membranes, and proteins. It involves four stages: G1, S, G2, and M, the G1 phase involves the synthesis of cellular organelles, DNA replication, organization and condensation of genetic

material, and the M phase, also known as the mitotic phase. This stage involves segregating duplicated genetic material into two distinct progeny cells. After the M phase, two daughter cells are generated, initiating a new cell cycle. To ensure accurate molecular process orientation, feedback control is necessary. If disrupted, apoptosis, a natural repairing process, results in programmed cell death (Roos and Kaina, 2006).

Checkpoint controls are crucial for maintaining chromosome integrity and completing essential cell cycle phases. They inhibit the activation of mitosis-promoting factors, such as cyclin and cyclin-dependent kinase (Cdk), during the S and G2 phases of the cell cycle. This prevents the activation of adenomatous polyposis coli and the initiation of anaphase. Mutations that prevent apoptosis can cause cells to become cancerous and proliferate rapidly. Natural substances can cause cell-cycle arrest in the G2-M, S, or G0-G1 phases(Alhmoud *et al.,* 2020).

ROS-mediated cancer cell apoptosis

Reactive oxygen species (ROS) are chemically reactive oxygen-containing molecules that play a crucial role in cellular signaling and homeostasis. They are generated as by-products of oxygen metabolism and can initiate programmed cell death, apoptosis, in cancer cells. Moderate increases in ROS can cause DNA mutations and inflammation, while excessive levels can lead to cell death through apoptosis, necrosis, and autophagy.

Research shows that reactive oxygen species (ROS) can induce apoptosis in cancer cells, potentially triggering cell cycle arrest, senescence, and death. Phytochemicals, such as those found in colon cancer, can trigger cell death through ROS generation, suggesting potential therapeutics for selectively targeting cancer cells by activating apoptotic pathways mediated by ROS(Ahmad *et al.,* 2019; Perillo *et al.*, 2020).

ROS-mediated apoptosis involves the activation of the intrinsic pathway, including mitochondrial permeability transition pores, cytochrome c release, Apaf-1, pro-caspase 9, and apoptosome formation. ROS can induce programmed cell death through modulating anti-apoptotic proteins like Bcl-2 and reducing pro-apoptotic molecules within the cell. This process culminates in the activation of effector caspases(Krishnan *et al.,* 2019; Nelson *et al.,* 2023).

Reactive oxygen species (ROS) are formed by risk factors associated with cancer, which trigger the activation of transcription factors like NF-kB, AP-1, HIF-1, and STAT3. These factors regulate cellular processes like inflammation, transformation, and metastasis. Some compounds, like polyphenols, can promote ROS formation through mitochondrial

mechanisms. Carcinogenesis is caused by excessive free radical generation, leading to DNA mutations and cancer progression. Antioxidants, both endogenous and exogenous, play a role in detoxifying free radicals and counteracting carcinogenesis. Plant-derived antioxidants with apoptosis-inducing properties have gained attention in cancer research due to their efficacy and cost-effectiveness (Gawlik-Dziki *et al*., 2013).

ROS-based anticancer medications have shown promising results in cancer treatment, as they activate programmed cell death through increased ROS levels, suggesting the potential for therapeutic interventions targeting ROS-induced apoptotic pathways.

Mitochondria-mediated apoptosis of cancer cells

Membrane potential refers to the difference in electric potential between a cell's interior and exterior. Mitochondria contain key caspase regulators, which are crucial in apoptotic processes. Mitochondria-mediated apoptosis is essential for programmed cell death, especially in cancer cells. The balance between pro- and anti-apoptotic Bcl-2 family proteins sets the threshold for mitochondrial apoptosis, which can be pore dependent or independent(Gilmore and King, 2019).

Studies have investigated mitochondria-mediated intrinsic apoptosis in cancer cells, focusing on cytochrome c dynamics and mitochondrial apoptotic pathways in lung and melanoma cancer. Targeting mitochondrial apoptosis is crucial for cancer therapy, as it initiates programmed cell death and is often linked to tumor development and drug resistance. Understanding and modulating this pathway is essential for developing targeted anticancer therapies(Gilmore and King, 2019).

Caspases and their role in cancer cell apoptosis

Caspases are crucial in apoptosis, acting as initiators and effectors. Three pathways trigger caspases: intrinsic, extrinsic, and granzyme B. Intrinsic caspases activate caspase-9, initiated by cytochrome c release from mitochondria; extrinsic caspases activate caspase-8, initiated by death ligands binding to cellular membrane death receptors; both pathways converge to caspase-3, which cleaves cellular components, leading to apoptosis(Wong, 2011).

Caspases are essential in apoptosis, a programmed cell death process, and dysregulation can prevent cancer cell death. Dysregulation of specific caspases can trigger aberrant cell survival, increasing tumorigenesis. Effector caspases facilitate apoptosis, but individual effector caspase failure as a cancer trigger is not widely described due to redundant functions during apoptosis execution (Olsson and Zhivotovsky, 2011; Boice and Bouchier-Hayes, 2020).

C-Myc protein in cancer

The c-Myc gene, a transcription factor, governs cellular processes like growth, differentiation, metabolism, and apoptosis. Dysregulation of this factor is common in human malignancies, and its ability to trigger apoptosis helps regulate and suppress uncontrolled cellular proliferation. Understanding the nature of c-Myc target genes is crucial for understanding its carcinogenic activation. The c-Myc gene is involved in cell cycle progression, apoptosis, and metabolism, and its dysregulation has been linked to the development of various types of tumors, such as breast cancer. Identifying direct targets of c-Myc target genes is common, but indirect targets could offer insights into the connections between dysregulated c-Myc expression and neoplastic transformation or apoptosis. Investigating c-Myc target genes is ongoing, and understanding the molecular mechanisms underlying c-Myc-induced apoptosis is essential for developing precise anticancer treatments(Ahmadi *et al.,* 2021; Gao *et al.,* 2023).

Over 50% of human malignancies have uncontrolled MYC family oncogenes, leading to poor prognosis and poor patient survival. C-Myc regulates fundamental cellular activities like differentiation, metabolism, G1-S transition control, apoptosis, proliferation, genomic integrity maintenance, and angiogenesis. MYC suppression is effective for treating various cancers, but direct targeting of Myc remains challenging due to its "undruggable" protein structure. Myc blockage alternatives include Myc/Max complex breakdown, Myc transcription/translation inhibition, Myc destabilization, and synthetic lethality linked to Myc overexpression(Chen *et al.*, 2018).

MYC activation in normal cells is self-controlled from malignancy through genetic and epigenetic checkpoints like mitotic arrest, apoptosis, and cellular senescence. MYC promotes stemness and inhibits cellular senescence and differentiation, while regulating tumor microenvironment changes like angiogenesis and immune system response. Myc is a key molecular player in carcinogenesis (Prochownik, 2004; Gabay *et al.,* 2014).

Literature review of the investigated plants

Plant description of *Olea dioica*

Kingdom : Plantae
Phylum : Tracheophyta
Class : Magnoliopsida
Order : Lamiales
Family : Oleaceae

Genus : Olea

Species : dioica Roxb.

Figure 5: Leaves of *Olea dioica* plant

In the present study, we have selected a medicinal plant, i.e., Olea dioica, which belongs to the Oleaceae family. *O. dioica*, commonly known as Rose Sandalwood, is a tree species native to the Indian subcontinent. It is found in both deciduous and evergreen forests, particularly in the Western Ghats of Peninsular India. The distribution of Olea dioica includes the Indian states of Maharashtra and Kerala, with its habitat ranging from semi-evergreen to moist deciduous forests at elevations of 150-1350 meters, as well as in the plains. This species is prevalent in the Western Ghats and is known for its self-supporting growth form, grey and smooth bark, and oblong-elliptic leaves that are pointed at both ends. The tree produces drupes and can reach heights of up to 15 meters. It is known by different names in different states, but in Karnataka it is known as Bilisarali, Edale, Kalluthodi, Tadale (Ashwathanarayana and Naika, 2017).

Traditional uses as medicine

The different parts of the plant have different uses; they are traditionally consumed to treat different diseases like fever, cancer, snake bite, rheumatism, skin diseases, anti-diabetic, neurodegenerative diseases and many more (Yesodharan and Sujana, 2007; Ashwathanarayana and Naika, 2017).

Phytochemical constituents of the plant

Gas chromatography and mass spectroscopy of crude *Olea dioica* ethanolic leaf extract showed the presence of different compounds, including gallic acid, caffeic acid, and quercetin (Ashwathanarayana and Naika, 2017a).

The methanol extract of *Olea dioica* leaves was found to contain flavonoids, triterpenoids, saponins, coumarins, and phenolic compounds such as gallic acid and quercetin. The study also identified eight phytocompounds using GC-MS analysis (Pratap and Shantaram, 2020).

The methanolic extract of *Olea dioica* leaves was found to contain various phytochemicals, including gallic acid and quercetin. The study also reported the presence of alkaloids, flavonoids, tannins, and phenolic compounds (Kekuda *et al.,* 2014).

The bark ethanolic extract of *Olea dioica* exhibited excellent radical scavenging and antimicrobial properties. The study highlighted the presence of phenolic compounds, including gallic acid and quercetin (Ashwathanarayana and Naika, 2015).

The study on the antioxidant and cytotoxic properties of *Olea dioica* crude extract and its pure compound identified the presence of triterpenoids, saponins, and coumarins (Ashwathanarayana and Naika, 2017b). These findings suggest that *Olea dioica* contains a diverse range of chemical compounds with potential bioactive properties.

Reported pharmacological activities

Antioxidant and cytotoxic properties: A study investigated the effects of the bark of *Olea dioica* and its isolated component. The bark was seen to display remarkable radical scavenging action, indicating its potential as a valuable reservoir of antioxidant and cytotoxic agents. (Ashwathanarayana and Naika, 2017b).

Antimicrobial and cytotoxic activity: The efficacy of *Olea dioica* extract was seen against various bacterial strains, namely *E. coli, S. aureus, S. flexneri*, and *B. cereus*, all of which are recognized pathogens associated with foodborne illnesses. The extract also exhibited activity against *Xanthomonas campestris* and *Ralstonia solanacearum*, which are plant harmful bacteria. Additionally, the extract exhibited cytotoxic activity against HT-29 and MDA-MB-231 cells (Kekuda *et al.,* 2014)

Analgesic Activity: Research focused on the analgesic activity of *Olea dioica* leaf extract, indicating its potential in pain management (Kekuda and Raghavendra, 2014).

Anticholinesterase Activity: The methanol leaf extract of *Olea dioica* exhibited antioxidant properties and acetylcholinesterase inhibition activity, suggesting its potential for neurological and cognitive health (Pratap and Shantaram, 2020).

Neuroprotective Potential: *Olea dioica* has been reported to exhibit neuroprotective effects, potentially offering benefits in the context of neurodegenerative diseases such as Alzheimer's

disease. The plant extract showed potential anti-AChE and anti-amyloidogenic properties, indicating its promise for creating new drug leads for controlling the progression of Alzheimer's disease (Kenchappa *et al.,* 2023).

Taking into consideration the various medicinal properties exhibited by this plant, it was decided to investigate the anti-cancer activity of a methanolic extract obtained from the leaf of *O. dioica.* Understanding molecular mechanisms, especially cell-based selective targeting, remains limited. Thus, in this study, we evaluated the molecular mechanism(s) that elucidate the selective anticancer activity of *O. dioica* leaf methanolic extract by employing A549 (human lung cancer), HCT116 (human colorectal cancer), HeLa (human cervical cancer), MCF-7, and MDA-MB-231 (human breast cancer).

The Western Ghats feature a lot of tree diversity and endemism. As a result, this study used the maceration extraction method to explore the accrual of bioactive components such as alkaloids, flavonoids, isoflavonoids, and phenolics in the leaves of *O. dioica*, as well as their anti-microbial activities. GC-MS screening was done to investigate the chemical compositions of bioactive substances in these variants. Despite decades of research, several highly significant medicinal herbs belonging to the genus *Olea dioica* that have been historically utilized by tribes and folklore remain unknown. Therefore, the objective of the study was to offer information on the expression of secondary metabolites in *Olea dioica* and its antibacterial and anticancer properties collected from the Western Ghats in Karnataka.

Objectives

- Collection of a sample and extraction of crude from the leaves of *Olea dioica* using methanol as a solvent
- Screening of phytochemicals qualitatively and isolation of secondary metabolites
- Estimation of cytotoxic activity in both crude and isolated metabolites
- Evaluation of cell apoptosis (expression of intrinsic and extrinsic pathway markers) induced by crude and isolated metabolites
- Study of mitochondrial membrane potential, ROS level and DNA damage in the MCF-7 human breast cancer cell line
- Evaluation of C-Myc protein expression after drug treatment

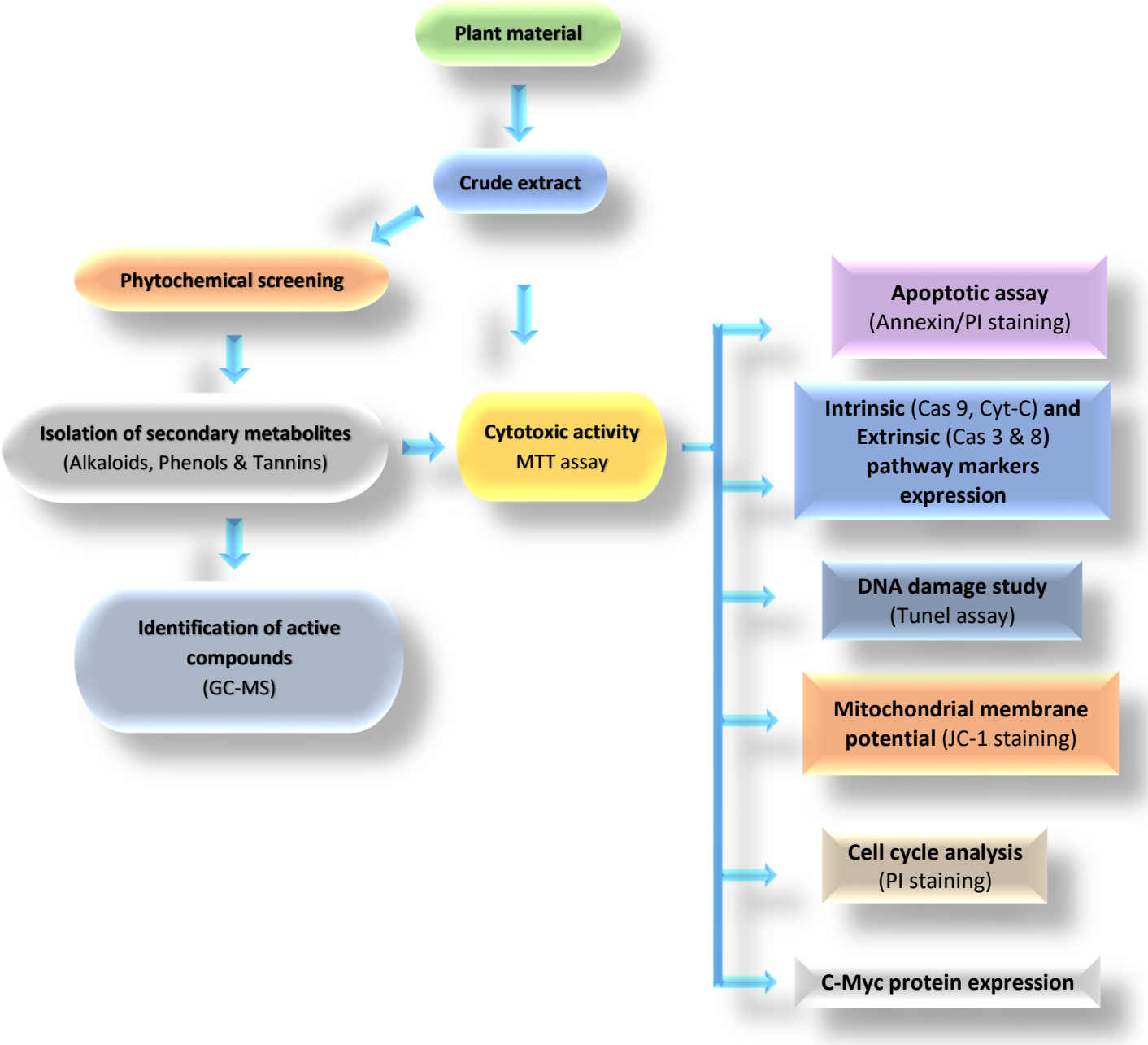

Fig. 2: Flow chart of the study

Collection of plant material

The leaves of *Olea dioica* were harvested from the eastern slopes of the Western Ghats in Kodagu, Karnataka, India, in the month of January 2019. The leaves of *Olea dioica* were subjected to washing using distilled water and pulverised using a machine. Finally, the powder was separated by sieving and stored for future use.

Preparation of plant extract

The process of preparing plant extract involves transferring a 100 g desiccated leaf powder sample into a vessel, adding 500 ml of methanol, and submerging the material in the solvent. The container is then kept at 37°C for 72 hours, repeating the process until a clear, colorless solvent is produced. The extracted mixture is separated from insoluble matter using Whatman No. 1 filter paper and evaporated in a water bath to obtain a concentrated extract. The extract is stored at 4°C for future use (Ujang *et al.,* 2013; Alam *et al.*, 2017; Ogbole *et al.,* 2017) .

Qualitative phytochemical screening of crude extract

The study focuses on the detection of various compounds in plant extracts. The tests include Dragendorff's test, Mayer's test, Wagner test, Molisch test, Liebermann-Burchard's test, Salkowski test, saponins test, Benedict's test, flavonoid test, ferric chloride test, lead tetra acetic acid test, and Keller Killani test.

The Dragendorff test involves agitating a predetermined amount of plant extract with 1% aqueous hydrochloric acid (HCl) in a water bath, followed by filtration. A good result is indicated by the presence of an orange-red precipitate. The Mayer's test detects the presence of certain functional groups, such as aldehydes, by adding a solution of mercuric chloride and potassium iodide to the mixture. The Wagner test involves the combination of 1 ml of extract with 1 ml of Wagner's reagent, which results in a reddish-brown precipitate (Sorescu *et al.,* 2018).

The Molisch test involves adding alcoholic alpha-naphthol solution to the extract and then introducing concentrated sulfuric acid (H_2SO_4). A violet colored ring emerges at the interface of two liquid phases, indicating the presence of carbohydrates (Kancherla *et al.,* 2019). The Liebermann-Burchard's test involves treating the plant extract with chloroform followed by filtration, followed by acetic anhydride, heating, and cooling to ambient temperature (Sharma *et al.*, 2015). The Salkowski test shows a reddish brown coloring, indicating positive outcomes regarding the presence of terpenoids (Harborne, 1998).

The foam test detects saponins by diluting the plant extract with distilled water and subjecting the mixture to agitation for 15 minutes. The detection of saponins is evidenced by the formation of a foam layer measuring roughly 1 cm.

The Benedict's test detects reducing sugar by boiling a volume of 5 millilitres of the extract with sodium carbonate, sodium citrate, and copper sulphate pentahydrate. The initial stage has a green coloration, followed by a precipitate that may be red, yellow, or green.

The flavonoid test indicates the presence of flavonoids by a yellow color. The ferric chloride test suggests the presence of phenolic compounds and tannins by observing a dark blue coloration (Sajid *et al.,* 2016). The Keller Killani test adds glacial acetic acid to one micro liter of the extract, followed by adding $FeCl_3$ and concentrated sulfuric acid (Archana *et al.,* 2012).

Antimicrobial activity

Test organisms

The study selected nine pathogenic bacterial test organisms from NCIM and IMTECH Chandigarh, including *Xanthomonas campestris, Pseudomonas syringae, Agrobacterium tumefaciens, Klebsiella pneumoniae, Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Streptomyces pneumoniae*.

***In-vitro* antimicrobial activity**

The antimicrobial activity of *O. dioica* methanol extract was tested against various pathogenic bacteria using a well diffusion assay. Bacterial collections were introduced into sterile nutritional broth tubes and incubated at 37°C for 24 hours. Test bacteria cultures were established by inoculating swabs onto nutrient agar plates. Wells with a diameter of 6 mm were made using a cork-borer. The methanol extract was dissolved in 1% DMSO and tested for antimicrobial activity in triplicate against various bacterial strains. Ciprofloxacin was used as the standard. The plates were maintained at ambient temperature for one hour and incubated at 37°C for 24 hours. The diameter of the inhibitory zone was measured using the Hi-media zone scale. The results showed that the methanol extract effectively inhibited various pathogenic bacteria (Karthik and Kalyani, 2022; R *et al.*, 2014).

Isolation of secondary metabolites

Alkaloids

A dried crude extract from a plant was dissolved in methanol, ammonia T.S. added, and stirred. The extract was treated with hydrochloric acid and filtered. The filtrate was then

alkalized and transferred to a separating funnel. Chloroform was introduced, and the mixture was allowed to undergo phase separation. The lower stratum contained alkaloids, while the upper stratum consisted of aqueous parts. The upper stratum was removed until the final chloroform extract yielded a negative result. The chloroform extract was partially dried, and the percentage yield was determined by dividing the weight of the alkaloid residue by the weight of the plant crude extract.

Phenols

Take 5gs of plant crude extract. Prepared the solvent by using ethanol, acetone, and water in a 35:1:14 ratio for 50 ml. 3. Mixed the above components by stirring at 50°C for 30 min. Centrifuged the mixture at 2500 rpm for 10 min. After centrifugation, took out the supernatant and dried it. The residue was quantified by measuring its weight, and the percentage yield was determined by applying the following formula:

% yield = (weight of the phenolic residue)/ (weight of plant crude extract) x 100

Tannins

The process involved solubilizing five grams of plant crude extract in methanol, centrifuging it at 11,000 rpm for 10 minutes, and adding 2M potassium dihydrogen phosphate to the supernatant. This precipitate was then rinsed three times with methanol and once with 1.2 N hydrochloric acid, stirring for a few hours. A saturated sodium carbonate solution was introduced into the tannin solution, calibrated to a pH of around 4.0. The lyophilization process was used to obtain a powdered form of dried tannin. The residual substance was measured in weight, and the proportion of the total yield was determined using the mathematical expression:

% yield = (weight of the tannin residue)/ (weight of plant crude extract) x 100.

Gas chromatography-mass spectroscopic analysis of O. dioica leaves

The study used a PekinElmer Gas Chromatograph Clarus 680 and a PekinElmer Mass Spectrometer Clarus SQ 8C to analyze the plant leaf extract of *O. dioica*. The GC-MS analysis was performed using a PekinElmer Elite-5MS standard column with a 30m length, 0.250mm inner diameter, and 1 micron particle size. The column operated within a temperature range of 60 to 350°C. The injected volume of 2 µL was 26.6 minutes, with a split ratio of 10:1. The carrier gas used was helium. The experimental conditions involved setting the source temperature to 230°C and the input temperature to 250°C. The oven temperature was initially set at 80°C, then increased to 150°C, 250°C, and 10°C. The components were identified through a comparative analysis with the components listed in the NIST computer

library integrated with the GC-MS apparatus. The results of this analysis were documented (Ukwubile *et al.,* 2019; Devi *et al.,* 2020) .

In vitro anticancer activity against cell lines

Cell lines and culture conditions

A subculture of cervical, colon, breast, and lung cancer cells was trypsinized in Dulbecco's Modified Eagle's Medium (DMEM) after removal. A volume of DMEM supplemented with 10% FCS was introduced to the disaggregated cells, which were gently suspended and homogenized using a pipette.

Cell culture

The cytotoxicity of *Olea dioica* plant crude extract was tested on five cell lines using DMEM supplemented with FBS, L-glutamine, gentamicin, penicillin, and streptomycin. The cells were seeded on 96-well cell culture plates and allowed to adhere for 24 hours. After washing twice with PBS, cells were treated with 0.25% trypsin/1 mM EDTA and then inactivated with new serum-containing media. A homogenized cell suspension was introduced into each well of a 24-well culture plate, and the plate was placed in a humidified CO2 incubator at 37°C with a CO2 concentration of 5%. After 48 hours, cells were examined using an inverted tissue culture microscope. A cytotoxic experiment was conducted using cells exhibiting 80% confluence, demonstrating the potential of *Olea dioica* plant crude extract in cytotoxicity (Sharif *et al.,* 2017; Soltanian *et al.,* 2017).

Cytotoxic MTT assay

The MTT assay, first reported by Mosmann *et al.,* in 1983, is a colorimetric technique used to assess cell viability and cytotoxicity. It relies on cells' ability to enzymatically decrease MTT salt, forming a formazan product. MTT is solubilized in aqueous solutions and undergoes cleavage by mitochondrial enzymes, transforming it into purple formazan, an insoluble crystal-like substance. This assay is widely used for evaluating cell proliferation and cytotoxicity (Mosman *et al.,* 1983).

The methyl-thiazolyl tetrazolium (MTT) assay was used to evaluate the cell viability of five different cell lines: A549 (human lung cancer), HCT-116 (human colorectal cancer), HeLa (human cervical cancer), MCF-7, and MDA-MB-231 (human breast cancer). The assay involved treating cancer cells with methanolic crude extract test compounds at various concentrations, with quercetin serving as a reference. The plates were incubated for 24 hours at 37°C with 5% carbon dioxide, then washed twice with phosphate buffer saline (PBS) to isolate spent media (Alam *et al.,* 2017). 10 µl of MTT reagent was added to a total volume

concentration of 0.5 mg/ml and incubated for 3 hours, wrapped in aluminium foil to prevent light exposure (Razak *et al.,* 2019).

The cells were then washed twice to remove MTT reagent and 100 µl/well DMSO was added as a solvent to solubilize the insoluble crystalline formazan products. The absorbance measurement was conducted using an enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 570 nm. The half-maximal inhibitory concentration (IC50) was calculated using the linear regression equation, and the viability graph was used to obtain the y = 50, m, and c values. The standard error of the mean was determined by quantifying the percentage of growth inhibition in test cells relative to control cells (Adeniran *et al.,* 2020).

Apoptosis assay by FITC annexin V/PI staining

The study followed the Annexin V-FITC Apoptosis Detection Kit protocol, which involved cultured cells in a 6-well plate and incubated for 12 hours in a CO_2 incubator. The cells were then treated with experimental drugs and controls, and then incubated for 24 hours. After treatment, the medium was extracted, rinsed with phosphate-buffered saline (PBS), and the sample was incubated at 37°C for 3 to 4 minutes. The cells were then harvested into a polystyrene tube, centrifuged, and decanted. A volume of 5 µL of FITC annexin V was introduced, and the cells were vortexed and incubated for 15 minutes at room temperature under darkness. Propidium iodide (PI) and 400 µL of 1X Binding Buffer were added to each tube, and the samples were subjected to flow cytometry analysis. The results showed that cells that had undergone mechanical damage, were in the late stage of apoptosis, exhibited a dual negative/normal phenotype, and were in the early stage of apoptosis were observed in the upper left, upper right, lower left, and lower right quadrants of the flow cytometric dot plot. The study provides valuable insights into the apoptosis process in cells (O'Brien and Bolton, 1995; Rieger *et al.,* 2011).

Mitochondrial membrane potential assay

MCF-7 cells were cultured in a T25 flask and incubated overnight at 37°C. After 24 hours of treatment with experimental compounds and controls, deceased cells were collected and transferred to polystyrene tubes. The cells were washed twice with PBS and centrifuged for five minutes. Each pellet containing less than 1 × 106 cells was treated with 0.5 ml of JC-1 working solution. The mixture was gently vortexed to disrupt cell pellets and prevent cell-to-cell clumping. The cells were incubated in a JC-1 working solution for 10-15 minutes at 37°C. In the first washing step, 2 ml of 1× assay buffer was added to each tube, and the cells were gently resuspended. The sample was centrifuged at 400 times the acceleration due to gravity

for 5 minutes. The cells were then resuspended in 0.5 millilitres of assay buffer with a concentration of 1×. The cells were then analyzed using flow cytometry.

Estimation of intracellular Reactive Oxygen Species (ROS) liberation

The study assessed the effect of *O. dioica* extract on reactive oxygen species (ROS) generation in cells. Cells were exposed to the extract for 24 hours, then trypsinized, washed, and resuspended in PBS supplemented with 10 μM DCFDA. Flow cytometry was used to analyze ROS expression, and cell QuestPro software was used for further data analysis.

Apoptosis by TUNEL Assay

TUNEL Protocol:

The APO-DIRECT™ Kit involves the suspension of cells in a solution of 1% paraformaldehyde in PBS (pH 7.4), followed by cooling on ice for 30-60 minutes, centrifugation at 300 x g for 5 minutes, and washing with PBS. The cells are then resuspended in phosphate-buffered saline and resuspended in a solution containing 70% ice cold ethanol. The cells are stored at -20°C until ready for use, with optimal results achieved 12-18 hours before staining. The cells can be preserved at -20°C for extended periods.

The staining protocol involves quantifying apoptosis in the positive and negative control cells. The cells are resuspended, and aliquots of the suspensions are extracted and transferred into centrifuge tubes. The suspensions are centrifuged at 300 x g for 5 minutes, and the 70% ethanol is removed to ensure the cell pellet remains undisturbed. The tubes are resuspended with wash buffer, and the sample is centrifuged again. The resulting supernatant is discarded.

Cytochrome-C expression involved in apoptosis

Cells were cultured in 6-well plates and incubated overnight in a CO_2 incubator at 37°C. After treatment, the cells were aspirated and treated with a concentration of the experimental substance. The medium was then washed with phosphate-buffered saline (PBS) and a toxicant concentration of 23 μM was added. The mixture was incubated at 37°C for 24 hours. The medium was then washed with PBS, trypsin-EDTA solution, and collected into a polystyrene tube. The tube was centrifuged at 300 x g for 5 minutes at 25°C. The supernatant was decanted, and the sample was treated with 70% ethanol. The cell pellet was stirred to immobilize the cells and minimize clumping. The sample was incubated at -20°C for 30 minutes and centrifuged for 5 minutes at a velocity exceeding the suitable cell viability. The supernatant was carefully removed without causing any loss of the pellet. The samples were

washed twice with PBS, and a volume of cytochrome c monoclonal antibody was introduced. The cells were then analyzed using flow cytometry on the FL1 channel.

Activity of Caspase 9

Cells are grown in 6-well plates in a CO_2 incubator at 37°C overnight. After 24 hours, they are incubated with various chemicals, aspirated, washed, and treated with toxicants. The cells are harvested into a polystyrene tube. The tube is centrifuged for 5 minutes, decanted, fixed with 70% ethanol, and pelleted. The cells are then incubated at -20°C for 30 minutes. The cells are then incubated with anti-caspase 9 antibodies in the dark for 30 minutes before being analyzed using FACS on the FL1 channel.

Caspase 3 expression of apoptosis by fluorescent microscopy

The study involved seeding MCF-7 cells in a 96-well plate and incubating them for 12 hours. After exhausting the medium, the cells were treated with IC_{50} concentrations of S1, S2, and STD for 24 hours. After washing, they were fixed and permeabilized using 70% methanol. After being rinsed with PBS, they were treated with anti-Caspase 3 -FITC antibody for 60 minutes. The cells were then visualized using a fluorescence microscope and analyzed using the ImageJ programme.

Caspase 8 expression by RTqPCR

The study involved seeding two ml of cell suspension in a 6-well plate, allowing them to grow for 12 hours. The cells were pre-treated with IC_{50} concentrations of test compounds and incubated for 24 hours at 37°C in a CO_2 incubator. After 24 hours, the cells were collected and used for RNA isolation. RNA was isolated using the Qiagen RNeasy kit, treated with DNAse, and purified to avoid genomic DNA contamination. The RNA was quantified by UV-Vis using Qiaexpert and quality checked by 260/280.

The cultured cells were treated with the IC_{50} concentration of the test substances and incubated in a carbon dioxide atmosphere for 24 hours. The cells were washed with phosphate-buffered saline (PBS) and the total RNA was isolated using the Quiagen Rneasy kit. The RNA concentration was assessed by quantifying the absorbance at a wavelength of 260 nm.

The cDNA synthesis was performed using the Biorad iscript cDNA synthesis kit, employing a combination of random hexamer and oligodT primers. The reaction volume for real-time PCR was set at 25 µl, consisting of 12.5 µl of SYBR mix and 9.5 µl of water. The forward primer and reverse primer were used in the qPCR reaction. The relative gene expression was determined

by comparing the CT value of the gene of interest with that of GAPDH, which serves as an internal reference.

Cell cycle regulation

The study used the propidium iodide (PI) staining method to analyze DNA in cells. The cells were grown in 6-well plates and incubated for 24 hours in a carbon dioxide incubator. The spent media was cleaned with PBS and exposed to the test chemical. After treatment, the cells were retrieved and stored in a 5 mL storage flask. The sample was centrifuged at 1800 revolutions per minute for 5 minutes at room temperature. The cells were then treated with 100% ethanol, which was pre-chilled in a freezer for 1 hour. The cells were then subjected to a second round of centrifugation to remove any remaining ethanol. To eliminate surplus ethanol, the cells were rinsed with PBS and centrifuged again. The cell pellet was treated with 400 μL of propidium iodide/RNase staining buffer to selectively stain DNA. The cells were incubated for 15-20 minutes at ambient temperature, shielded from light. The samples were then subjected to flow cytometry analysis in a solution containing propidium iodide (PI) and RNase, without the need for cell washing.

C-Myc protein expression

Cells were cultured in 6-well plates and incubated overnight in a CO_2 incubator at 37°C. After aspiration, the cells were treated with a concentration of the experimental substance and incubated for 24 hours. The medium was then washed with phosphate-buffered saline (PBS) and a hazardous agent. The mixture was then incubated at 37°C for 3-4 minutes. The cells were harvested into a polystyrene tube and centrifuged for 5 minutes at 25°C. The supernatant was then rinsed with PBS and treated with 70% ethanol to immobilize the cells. The sample was incubated in a freezer at -20°C for 30 minutes and centrifuged for 5 minutes at a higher speed to allow live cells to remain suspended. The supernatant was carefully removed without disturbing the pellet. The cells were then treated with a monoclonal C-MYC antibody and incubated under ambient conditions for 30 minutes. A volume of DPBS was added, and the cells were analyzed using FACS on the FL1 channel. The process involved a series of steps to ensure accurate results.

Statistical analysis

The result obtained was shown as mean SD (standard deviation). The MTT test was conducted in triplicate. The statistical data was shown as standard error of the mean. The statistical software GraphPad prism version 9.0 for windows was used. The Tukey's test was

used along with one-way ANOVA (Analysis of variance). The significance level was set at $p < 0.05$. The IC_{50} values were calculated from linear regression analysis (Soltanian *et al.,* 2017).

Preliminary phytochemical screening

The study focuses on the bioactive compounds found in the plant sample of *O. dioica* from the eastern slopes of Western Ghats, which serves as an alternative to conventional antibiotics and therapeutics. Methanol solvent was used for extraction due to its strong polarity and potential for high yields. The methanolic leaf extract of *O. dioica* contains phytochemicals such as alkaloids, sterols, terpenoids, flavonoids, and phenolic compounds, which have been evaluated for their potential in treating various ailments, including antibacterial and anticancer (Borges *et al.,* 2020).

GC-MS analysis

The GC-MS analysis of the methanolic extract of *O. dioica* leaves revealed 38 peaks corresponding to various chemical compounds. The highly probable bioactive compounds were characterized using GC-MS, with prominent chemical constituents including Octanoic acid, methyl ester (66.8%), Decanoic acid, methyl ester (44%), Octanoic acid (36.3%), Desulphosinigrin (15.4%), Methyl tetradecanoate (63%), Tridecanoic acid, 12-methyl-methyl ester (18.5%), Tetradecanoic acid (49.5%), 6-benzoxazolesilfonamide, N-(2-hydroxyethyl)-2-methyl ester(34.3%), 10-chloro-5-methoxy-5H-dibenzo annulene (29%), Pentadecanoic acid (23.7%), Oleic acid (30.8%), n-Hexadecanoic acid (60.1%), 9-Octadecenoic acid (Z), methyl ester (17.6%), Hexanedioic acid, dioctyl ester (18.9%), and Squalene/Supraene (28.2%).

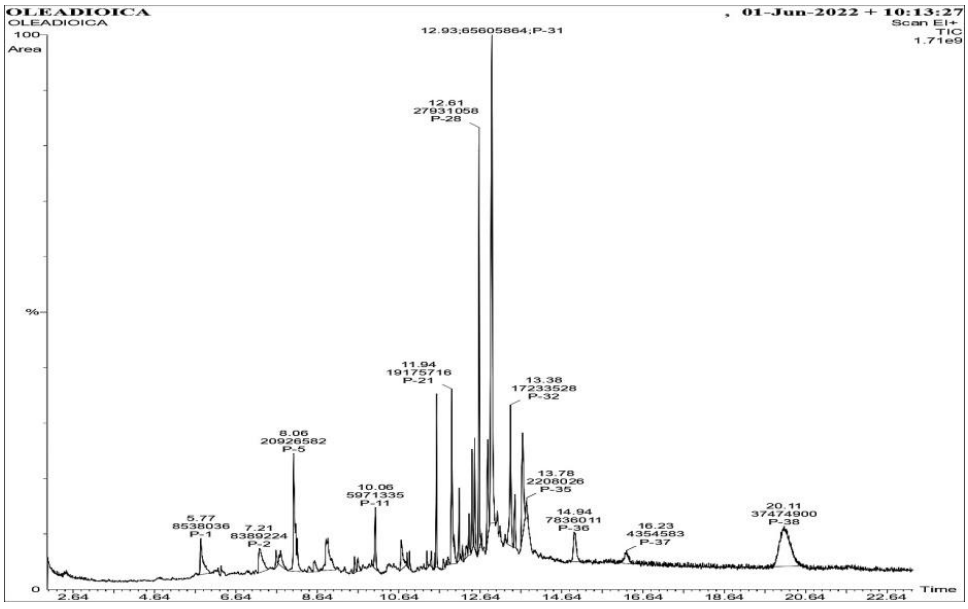

Figure 1: TIC (Total ion chromatogram) of GC-MS for methanolic leaf extract of *O. dioica*

The GC-MS analysis of 118 bioactive phytochemical compounds revealed their biological activity. Octanoic acid methyl ester and similar compounds have been found to exhibit anticancer, anti-asthmatic, and anti-inflammatory properties (Sampathkumar *et al.,* 2020). Desulphoainigrin has anticancer, anti-asthmatic, and anti-inflammatory properties (Krishnaveni, 2015; Kadhim *et al.,* 2017) . l-Gala-l-ido-octose has potential in memory drug production for dementia prevention (Chukwu *et al.,* 2020). Tridecanoic acid, 12-methyl-, methyl ester, Hexanoic acid, 2-methyl-, Benzoic acid, 2-(4-methylphenoxy)-, methyl ester, and Tetradecanoic acid have antimicrobial properties against fungi and bacteria (Elaiyaraja and Chandramohan, 2018). Tetradecanoic acid serves as a flavoring agent and exhibits larvicidal and repellant properties against *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes (Maduabuchi and Awucha, 2020; Sivakumar *et al*., 2011). Melibiose, a reducing sugar, has potential therapeutic applications in polyQ diseases. Benzofuran-2-one, 2,3-dihydro-3,3-dimethyl-4-nitro-, 13-Tetradecynoic acid, and methyl ester have anti-inflammatory properties (Altaee *et al*., 2017). cis-Vaccenic acid has therapeutic applications in cardiovascular diseases (Field *et al*., 2009; Semwal *et al*., 2018),. Hexadecanoic acid has various beneficial properties, including anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne, alpha-reductase inhibitor, antiandrogenic, antiarthritic, and anticoronary properties (Ragupathi*et al.,* 2018). n-Hexadecanoic acid has antioxidant and anti-inflammatory properties, as well as hypocholesterolemic, nematicidal, pesticidal, lubricating, anti-androgenic, flavor-enhancing, hemolytic, and 5-Alpha reductase inhibitory effects (Aparna *et al.,* 2012; Maduabuchi and Awucha, 2020).

Antibacterial activity of methanolic extract of *O. dioica*

The methanol extract of *O. dioica* leaves was tested for its antimicrobial potency against nine pathogens, including *Xanthomonas campestris, Pseudomonas syringae, Agrobacterium tumefaciens, Klebsiella pneumoniae, Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, and Streptomyces pneumoniae*. The antibacterial efficacy was compared to the conventional antibiotic ciprofloxacin, with the zone of inhibition diameter measured in millimeters. The results are listed in Table 3.

Table 3: Antibacterial activity of *O. dioica* methanol extract against different pathogens

Pathogens	Zone of inhibition in mm				Standard 20 µg (Ciprofloxacin)
	25 µg	50 µg	75 µg	100 µg	
Pseudomonas aeruginosa	0±0	8±0.05	12±0.05	14±0.05	36±0.05
Escherichia coli	0±0	9±0.05	16±0.05	19±0.05	34±0.05
Klebsiella pneumoniae	8±0.05	10±0.05	14±0.11	18±0.05	37±0.05
Streptomyces pneumoniae	8±0.05	10±0.05	9±0.1	16±0.05	36±0.05
Pseudomonas syringae	0±0	7±0.05	11±0.1	14±0.05	32±0.05
Staphylococcus aureus	0±0	11±0.2	13±0.05	19±0.05	34±0
Xanthomonas campestris	0±0	9±0.3	14±0.1	18±0.05	31±0.05
Salmonella typhimurium	0±0	10±0.05	12±0.1	19±0.1	35±0.11
Agrobacterium tumefaciens	0±0	7±0.05	10±0.05	14±0.05	35±0.05

Isolation of secondary metabolites

Alkaloids are organic molecules found in various organisms, containing carbon, hydrogen, nitrogen, and oxygen. They are used in dietary products, supplements, and medications (Kurek, 2019). Extracting alkaloids from plant materials involves separating biologically active components from inert substances using a solvent and a standardized extraction protocol (Abubakar and Haque, 2020). Phenolic chemicals, found in plant species, are secondary metabolites with diverse structural diversities. Isolating phenolic compounds from plant materials is crucial for analyzing polyphenolic compounds, which are essential for determining percentage yield (Alara *et al.,* 2021). Tannins, a collection of polyphenolic compounds, are used in various fields, including feed for ruminants and monogastric animals Cuong *et al.,* 2019). The yields of methanol crude extract and isolated alkaloids, phenols, and tannins were found to be 30.72%, 1%, 1%, and 0.96%, respectively.

***In vitro* anticancer activity of methanolic leaf extract of *O. dioica* against human cancer cell lines**

Cytotoxic analysis of methanolic crude extract of *O. dioica* leaves

Cytotoxicity is the ability of a substance to cause cell death, and the MTT assay is a common method for measuring this(Memariani *et al.*, 2016; Ogbole *et al.,* 2017; Ala *et al.,* 2018). This study evaluates the cytotoxic potential of *O. dioica* methanol leaf extract on human cancer cell lines A-549, HCT-116, HeLa, MCF-7, and MDAMB-231. The extract showed increased inhibition with increasing concentration and morphological changes in treated

cells. The highest concentration used was 500 µg/ml, with IC_{50} values of 81.05 µg/ml, 106.86 µg/ml, 141.34 µg/ml, and 499.24 µg/ml against HeLa, MCF-7, HCT-116, and MDAMB-231 cell lines, respectively. Quercetin, an anticancer agent, was used to compare the effects of the leaf extract on cell viability to routinely used drugs.

The cytotoxic capability of *O. dioica* plant ethanol extracts on DLA and EAC in Swiss albino mice was tested using MTT assay (Ashwathanarayana and Naika, 2017c). The methanolic leaf extract showed high efficacy on HT-29 and MDAMB-231 cells, with IC_{50} values of 131.87 µg/ml and 140.25 µg/ml, respectively (Kekuda and Raghavendra, 2014). The extract also showed significant activity on MCF-7 human breast cancer and HeLa human cervical cancer cells, with IC_{50} values of 106.86 and 81.05 µg/ml, respectively. The HeLa cervical cancer cell showed the most cytotoxic effect, with an IC_{50} value of 81.05 µg/ml.

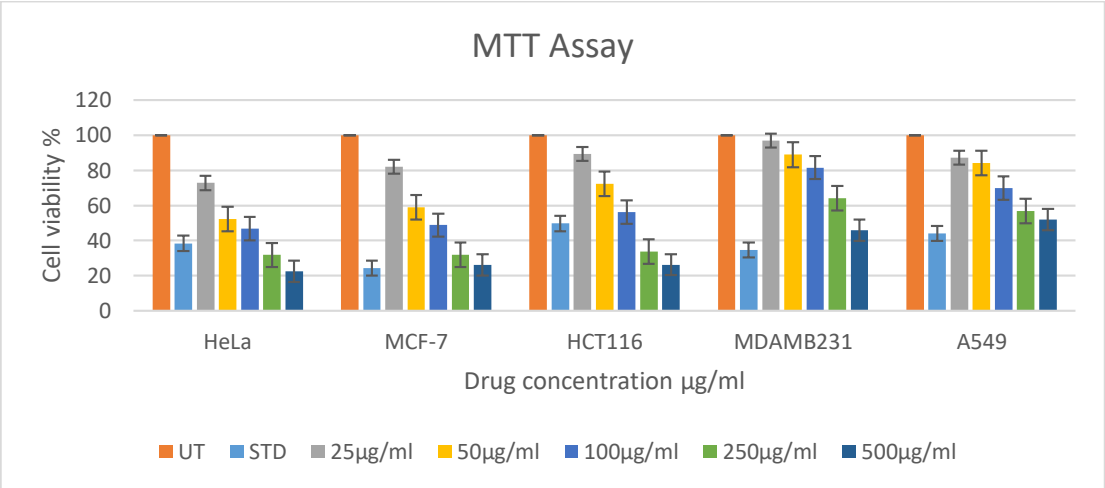

Figure 3: Cell viability percentage of *O. dioica* methanolic leaf extract against HeLa, MCF-7, HCT-116, MDAMB-231 and A-549 cancer cell lines at different concentrations by MTT assay.

Cytotoxicity analysis of secondary metabolites isolated from the methanolic extract of *o. dioica* leaves

The alkaloid extract of *O. dioica*'s methanolic leaf extract decreased cell viability against cancer cell lines, losing 50% of HCT-116 and MCF-7 cell viability at doses of 447.17 µg/ml and 315.58 µg/ml, respectively. However, cells like A-549, HeLa, and MDAMB-231 took more than 500 µg/ml doses to inhibit growth.

Alkaloids, chemically significant molecules, are valuable for the exploration and development of pharmaceutical agents. They have been studied for their potential anti-proliferative and anticancer properties against various malignancies, both *in vitro* and *in vivo* (Lu *et al.,* 2012; Mondal *et al.,* 2019). Alkaloids, including vinblastine, vincristine, vinorelbine, and vindesine, hold great promise for cancer treatment and control (Mondal *et al.*, 2019). This

study examines alkaloids derived from natural herbs like berberine, evodiamine, matrine, piperine, sanguinarine, and tetrandrine, due to their higher anticancer properties. However, current understanding of their mechanisms is limited *(Lu et al.,* 2012).

Cytotoxicity of phenols against cancer cell lines

Using the MTT assay, it was discovered that the phenol extract of the methanolic leaf extract of *O. dioica* decreased the viability of the cells against cancer cell lines as the dose increased (Figure 10). 50% of the viability of HCT-116 cells was lost at extract concentrations of about 478.53 µg/ml, in comparison to untreated cells (control) (Table 6). On the other hand, A-549, HeLa, MDAMB-231, and MCF-7 cells required a dosage of extract exceeding 500 µg/ml in order to prevent 50% growth suppression. According to results the phenols can inhibit only HCT-116 cells among all the five cancer cells.

Phenols, naturally occurring chemical molecules found in plants, have been linked to cancer prevention and treatment (Basli *et al.,* 2017). Studies have shown that phenolic compounds, including curcumin, baicalin, and phenolic acids, have significant inhibitory effects on cancer cell development. However, their effectiveness varies depending on their structure, molecular targets, and the type of cancer being treated (Jafari *et al.,* 2014). The administration of phenolic compounds for cancer treatment may impact biological processes, including preventing infectious and inflammatory reactions. Toxicity concerns have been reported, but progress has been made in medication administration and treatment methods to mitigate these issues. Despite their potential, further research is needed to fully understand their modes of action and potential adverse effects (Basli *et al.*, 2017; Abotaleb *et al.,* 2020).

Cytotoxicity of tannins against cancer cell lines

From the MTT cytotoxic assay of isolated phenols in the methanolic leaf extract of *O. dioica*, it was observed that the dose-response inhibition as the dose increased, cell viability decreased (Figure 11). The cancer cells MCF-7 and MDAMB-231 have shown inhibition from the very first dose but significantly 50% of cells died at 158.52 µg/ml and 306.57 µg/ml dose of methanolic leaf extract respectively. Contrarily, there is no discernible inhibition of A-549, HeLa, or HCT-116 cell viability observed after treatment with phenol extract (Table 7). In order to compare our results with a known anticancer medication, quercetin was utilized as a positive control.

Tannins, a type of polyphenolic compound, have shown promising potential in cancer prevention and treatment (Kleszcz *et al.,* 2023). They can impede cellular signaling pathways and oncogenic proteins that contribute to lung and breast cancer progression. Tannic acid

(TA) has shown targeted anticancer effects against lung and breast cancer (Rajasekar *et al.,* 2021). It facilitates programmed cell death in embryonic cancer stem cells through TRAIL-mediated apoptosis. Tannic acid also impedes breast cancer cell viability by interacting with cell surface growth factor receptors like EGFR, IGFR, and oestrogen receptors. In gingival cancer, TA down-regulates STAT3 signal and cell viability (Youness *et al.,* 2021). Overall, tannins like tannic acid have shown promise in cancer prevention and treatment (Sp *et al.,* 2020). Further research is needed to understand their mechanisms of action and develop novel cancer prevention agents or anticancer medicines.

The crude methanolic leaf extract of *O. dioica* and its metabolites, including alkaloids, phenols, and tannins, have shown significant inhibition against various cancer cell lines, including MCF-7. The IC_{50} values for crude against MCF-7 were 106.86 µg/ml, while tannins showed 158.52 µg/ml. This suggests tannins contribute significantly to the protective effects of crude against breast cancer, prompting further research on apoptosis' role in breast cancer prevention.

Annexin V and PI labelling for quantifying apoptosis

After incubating MCF-7 cells for 24 hours, it was discovered that S1 (Crude extract) and S2 (Tannin extract) cause 38.28% and 22.76% early apoptosis, 0.82% and 0.13% late apoptosis using Annexin V and PI staining (Table 8 and Figure 12). Whereas untreated cells have not undergone apoptotic cell death, but the standard used in the study quercetin has shown 63.65% of early apoptosis of MCF-7 cells. These findings demonstrate that, in comparison to S2, S1 is more adept in causing tumor cells to undergo apoptosis.

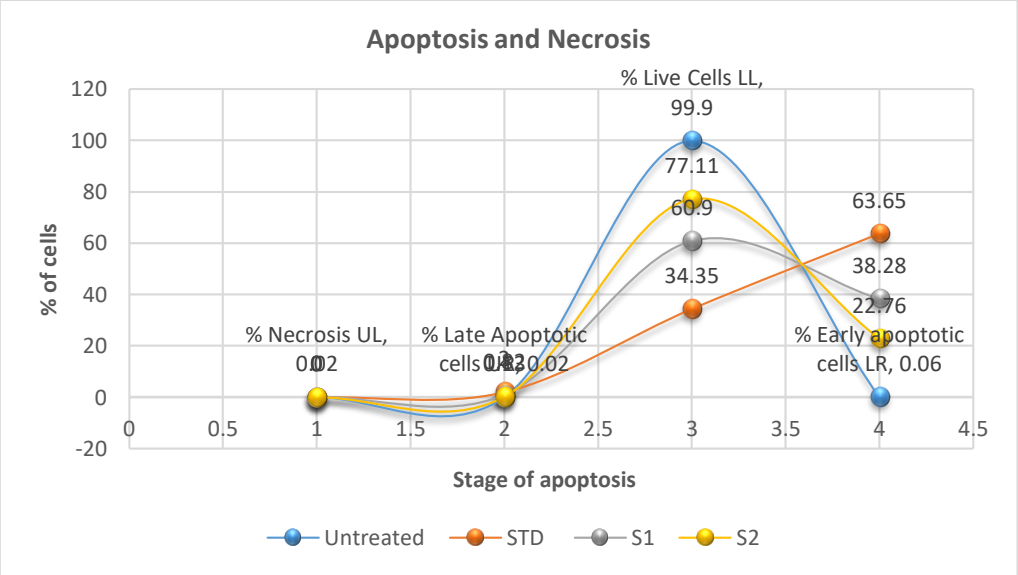

Figure 12: Apoptotic effect of methanolic leaf crude extract and isolated tannin extracts of *O. dioica* on cell lines MCF-7.

Annexin V and PI are used to identify cell death types like necrosis or apoptosis. The Annexin V/PI procedure is popular for examining apoptotic cells, with PI being more cost-effective and stable. Annexin V binds to phosphatidylserine on apoptotic cell surfaces, allowing researchers to detect apoptotic cells using flow cytometry. Cell labelling using Annexin V and PI allows researchers to assess cell viability based on changes in plasma membrane integrity and permeability.

Changes in mitochondrial membrane potential

The study found that cells exposed to S1 and S2 concentrations showed a significant decrease in JC-1 red fluorescence, indicating an elevation in the depolarization of the mitochondrial membrane potential. This was supported by a decrease in the mean fluorescence intensity of red fluorescence and an increase in the number of cells within the apoptotic gate, suggesting that the mitochondrial pathway may induce apoptosis.

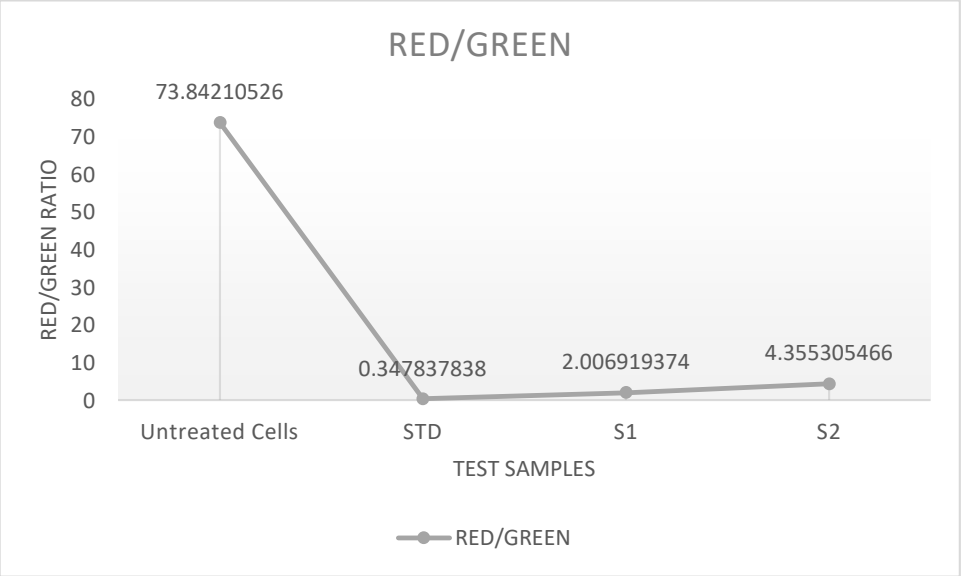

Figure 14: Mean ratios of percentage of Red/Green fluorescence expressing cells after 24hrs of incubation with standard, S1 and S2 samples.

Mitochondrial membrane potential (MMP) is a crucial aspect of cell health and functionality. During apoptosis, the MMP decreases, a process considered an initial step. JC-1, a cationic carbocyanine dye, can be used as a sensitive fluorescent probe to measure MMP in various cell types, including myocytes, neurons, intact tissues, and isolated mitochondria (Perelman *et al.,* 2012). The mitochondria of JC-1 show potential-dependent accumulation, as evidenced by a shift in fluorescence emission from 529 nm (green) to 590 nm (red). In normal cells, JC-1 exists in an aggregate form, producing red fluorescence. However, during apoptosis, the MMP decreases, causing JC-1 to be monomeric within the matrix, resulting in green

fluorescence. This change in color reflects the decline in MMP, making it a useful marker for early apoptosis detection (Perelman *et al.,* 2012; Sivandzade *et al.,* 2019).

Effect of plant extracts on the level of ROS liberation

In this investigation, it was found that treating MCF-7 breast cancer cells with extracts enhanced the formation of ROS. When treated with an IC_{50} dose of S1 and S2 extracts, we observed a considerable increase in the number of fluorescent cancer cells relative to the cancer control cells (Fig. 16). The finding that S1 extract works specifically and selectively on cancer cells is corroborated by the production of ROS release in cancer cells solely after treatment with S1 and S2 extracts.

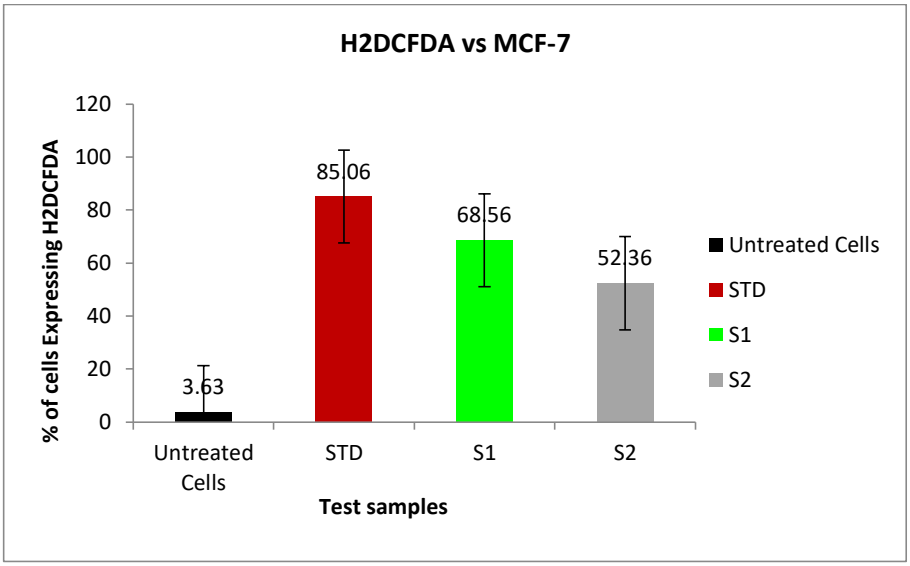

Figure 16: Measurement of intracellular ROS in MCF-7 cells

Reactive oxygen species (ROS) are molecules that can induce DNA mutations and programmed cell death (PCD) in cancer cells (Perillo *et al.,* 2020). They can trigger cell cycle arrest, senescence, and apoptosis through intrinsic or extrinsic apoptotic signaling pathways *(Krishnan et al.,* 2019). ROS can inactivate or upregulate Bcl-2, decrease intracellular levels of Bax and Bad, and regulate apoptosis (Khan *et al.,* 2021; Nelson *et al.,* 2023). Benzophenanthridine alkaloids, which induce cell death through ROS generation, have been found to induce cell death in cancer cells. Cancer cells counteract the accumulation of excessive ROS within their environment (Nakamura and Takada, 2021).

Cytochrome-C induced apoptosis in mitochondria mediated pathway

The fluorescence results of cells challenged with IC_{50} concentration of test compounds, which were subsequently labelled with an anti-cytochrome c antibody and analysed using flow cytometry were shown that S1 treated cells have released about 68.58 %

of cytochrome c whereas in the cells treated with S2 have shown 54.93 % of cytochrome c expression over control and untreated cells at time 24 h, about 86.05% and 3.02 % of expression.

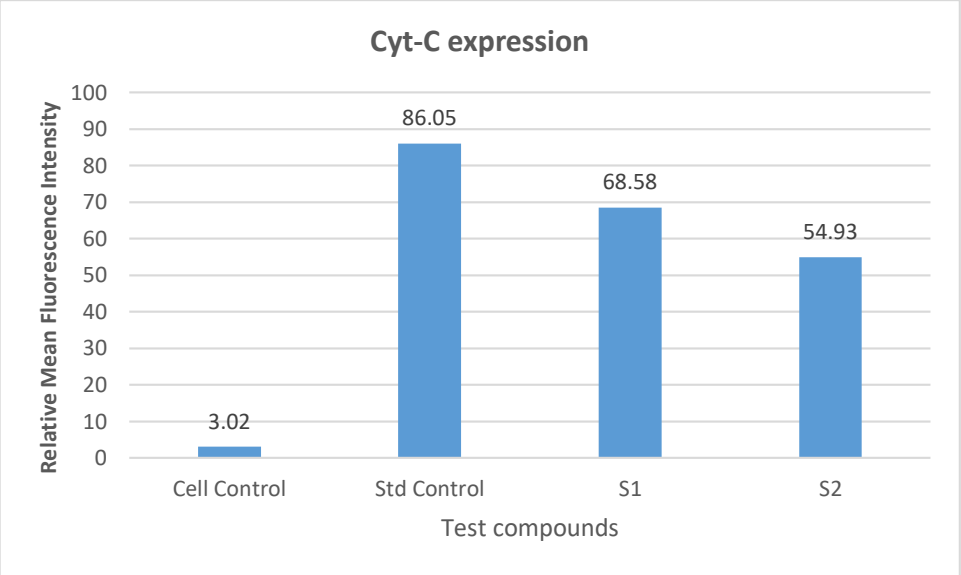

Figure 18: % of Cytochrome-C expressing cells treated with desired concentration of STANDARD, and IC_{50} concentration of S1, S2, Untreated cells used as negative control

DNA damage by TUNEL assay

The TUNEL assay was employed in conjunction with flow cytometry to assess the extent of DNA damage induced by the methanol crude extract, tannic extract of *O. dioica*, and the reference drug quercetin. The findings indicated that a significant proportion of cells (97.11%) treated with quercetin exhibited DNA damage. In a similar manner, the methanol crude extract (S1) exhibited a significant effect on MCF-7 cells, with a 92.81% inhibition rate seen at the IC_{50} concentration. The untreated cells and S2 cells demonstrated DNA damage in just 2.77% and 53.92% of cells, respectively.

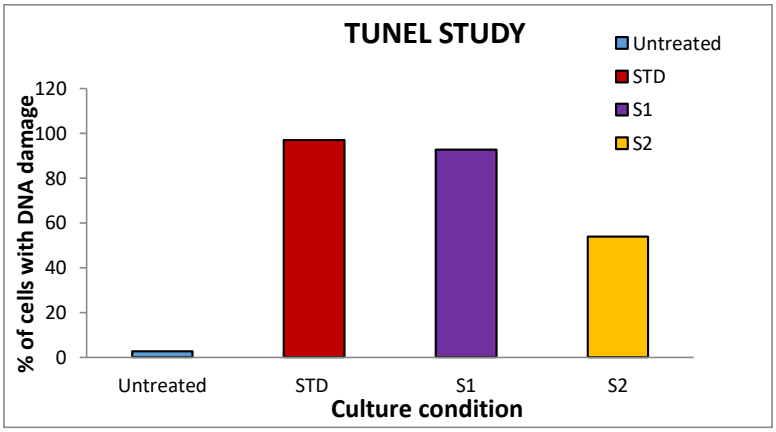

Figure 20: % of cells with DNA damage in Standard, Untreated, S1 and S2 against MCF-7 cancer cells

Caspase 8 mRNA expression by qRT-PCR

The qRT-PCR assay revealed that the mRNA levels of caspase 8 genes increased significantly by 8.36-fold change in S1 treated and 6.54-fold change in S2 treated MCF-7 cells over standard control 11.59 fold change. Untreated cells have shown only 1-fold change (Figure 22). If a fold change is more than 1 is up-regulation of gene and less than 1 is down regulation of a particular gene. Hence the result was observed in the up-regulation of a caspase 8 gene expression. This indicates the involvement of caspase 8 expression in apoptosis induced by S1 and S2.

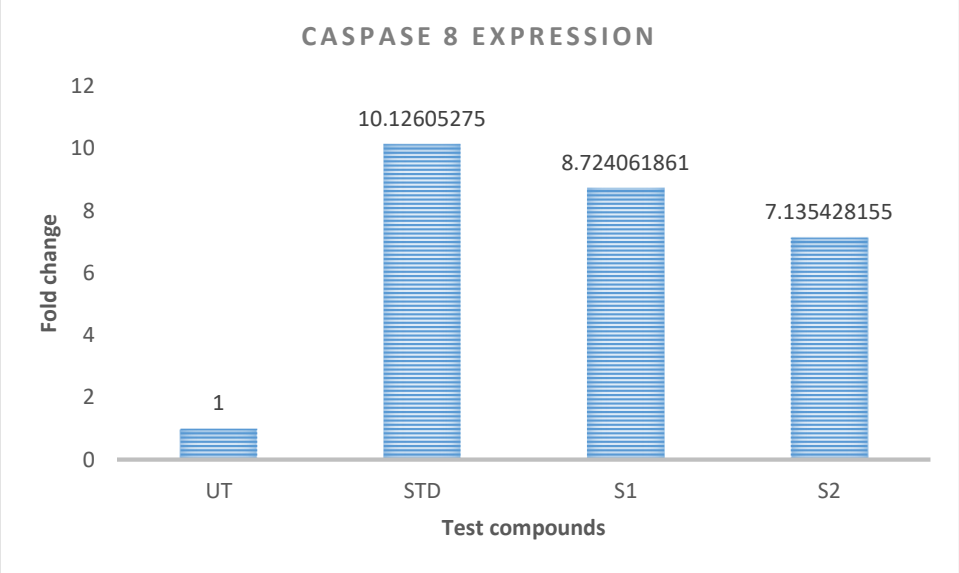

Figure 22: Fold change in expression of caspase 8 in apoptosis induced by S1 and S2 in MCF-7 cells.

Caspase 9 protein expression

The results obtained from the caspase 9 assay using flow cytometry demonstrated that the percentage of cells exhibiting caspase 9 release was approximately 70.89% and 58.36% in cells treated with methanol crude extract and isolated tannin, respectively. In contrast, cells treated with the standard drug quercetin exhibited a higher percentage of caspase 9 release at 88.48%, while untreated cells showed a significantly lower percentage at 2.92% (Figure 23). The data of the study indicate S1 show greater increase in caspase activity, when compared to S2 based on analysis by flow cytometry. The findings of *in vitro* investigations indicate the potential utility of S1 in the therapeutic management of breast cancer cells.

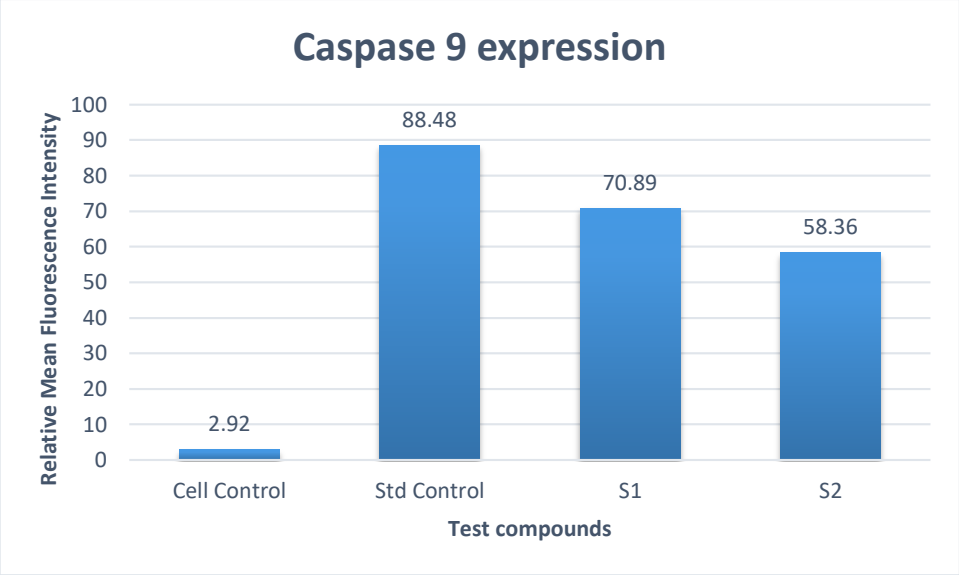

Figure 23: % of Caspase 9 expressing cells treated with desired concentration of STANDARD, S1, S2, Untreated cells used as negative control.

Expression of caspase 3 protein

The expression of caspase 3 protein through florescent microscope showed that the effect of S1 and S2 treated cells, about 31.1 and 22.74 % of mean green florescence, whereas in cells treated with standard drug quercetin was expressed about 37.69 % of caspase 3 and in untreated cells there was no observation of caspase 3 expression (Figure 24). The findings of this study provide compelling evidence that the growth-inhibitory effects of S1 and S2 can be linked to the induction of apoptosis via the activation of caspase-3.

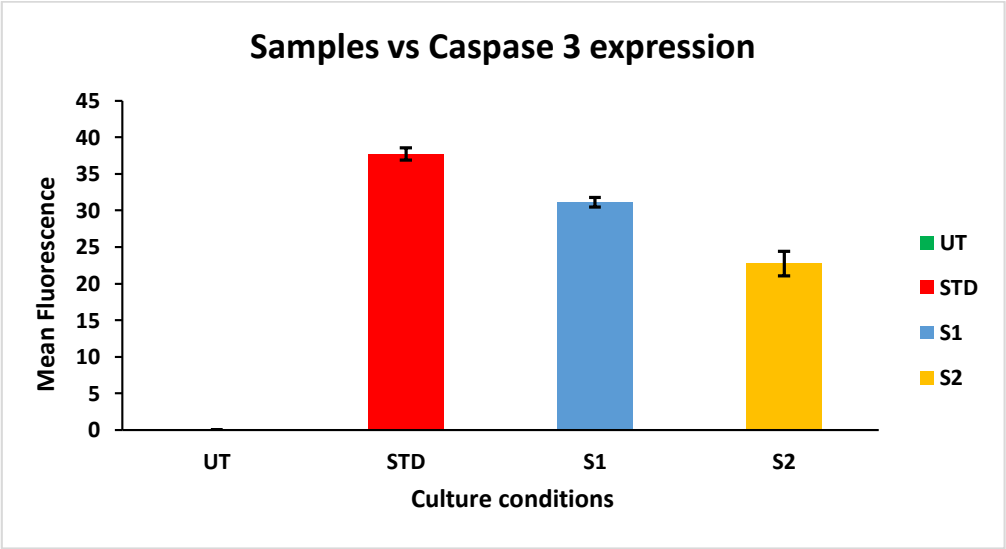

Figure 24: Mean green fluorescence intensities of Caspase 3 expression in MCF-7 cells treated with S1, S2 and STD for 24 hrs.

C-MYC oncogenic protein Expression by FACS

The flow cytometric assessment of C-myc protein expression which involves in the induction of apoptosis after treating with S1 and S2 against MCF-7 cells, it was determined that S1 and S2 induce 33.36 % and 42.73 % of C-myc gene suppression relative to standard 10.37 % and control 97.3 % of expression respectively (Figure 25).

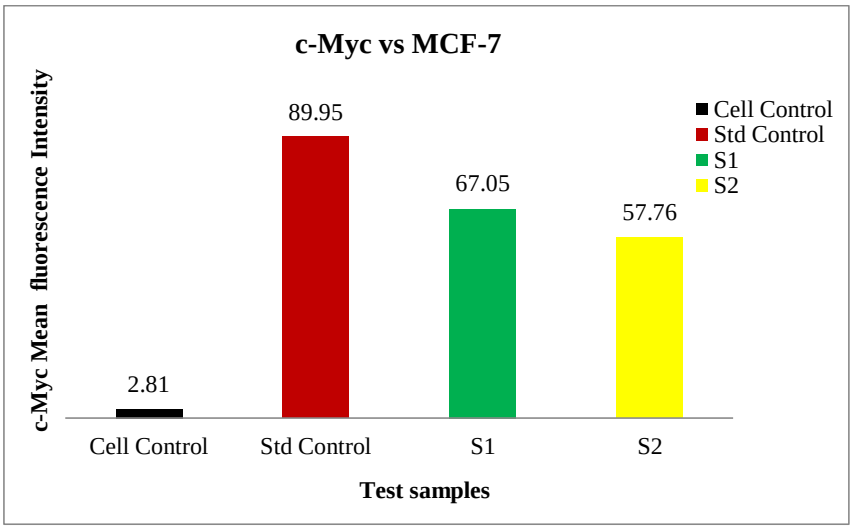

Figure 25: % of c-Myc protein expressing cells treated with desired concentration of STANDARD, S1, S2, Untreated cells used as negative control.

Methanolic leaf extract of *O. dioica* disturbed cell division cycle and induced G1 arrest

The provided samples were shown to impede the distribution of cancer cells throughout the cell cycle, as assessed by flow cytometry. These findings illustrate the distribution of the cell cycle in MCF-7 cells following a 24-hour exposure to plant extracts, as depicted in Figure 29. The application of the extract to MCF-7 cells led to a significant increase in the percentage of cells arrested at the G0/G1 phase, with values of 44.9% and 47.59% observed, compared to the control group where 47.36% of cells were in the G0/G1 phase. This arrest at the G0/G1 phase was accompanied by a reduction in the proportion of cells in the S phase and G2-M phase in the cancer cells treated with the extract, as depicted in Figure 28. Therefore, the application of S1 resulted in the suppression of cell development through mediation and induced arrest in the G0/G1 phase of MCF-7 cells.

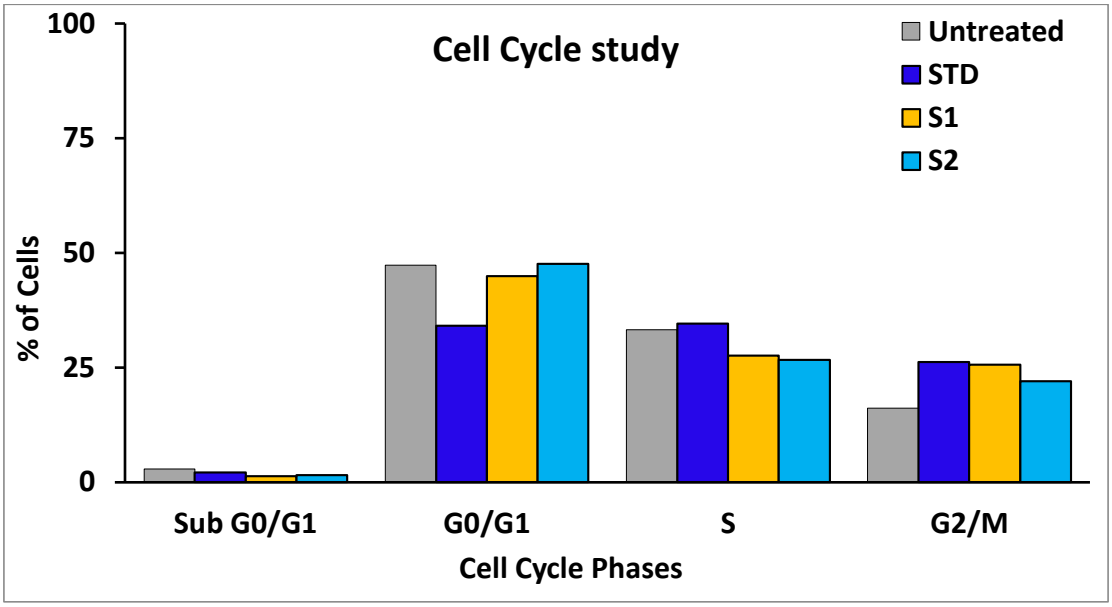

Figure 28: % of Cells get arrested in different cell cycle stages of MCF-7 cell line. Untreated cells used as negative control and Standard Quercetin as a positive control

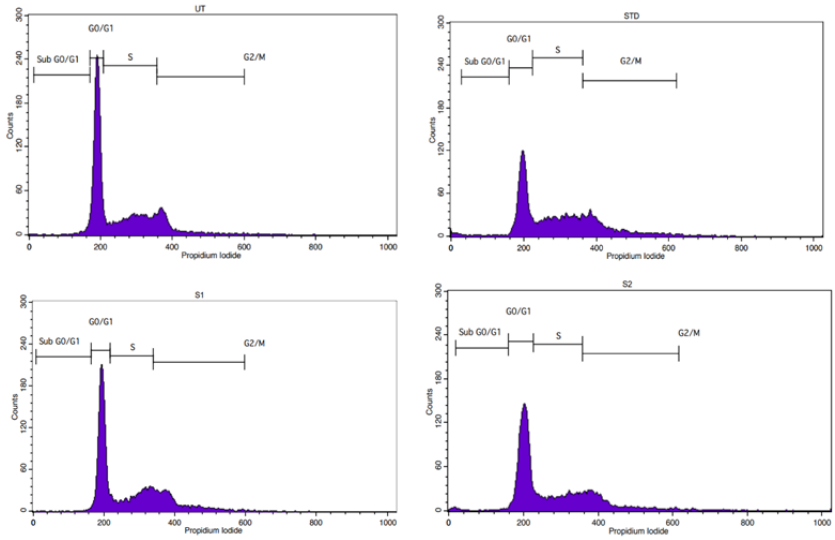

Figure 35: Flow cytometric histograms showing the phases of cell cycle distribution in MCF-7 cell line treated with IC50 values of S1, S2 and STD.

CHAPTER IV
SUMMARY AND CONCLUSION

Cancer is a class of disorders defined by uncontrolled cell division in one region of the body and the ability to infiltrate other tissues or parts of the body. It is the second-greatest cause of death worldwide. According to the global cancer incidence, lung, breast, colorectal, cervical, and prostate cancers are common in the population. Despite all the treatments available in the medical system, the research on finding treatment using natural resources are still in progress. In that context this study chose a medicinal plant *Olea dioica* Roxb., which has potent medicinal properties including the anticancer activity. Hence the methanolic crude extract of *O. dioica* leaf was compared with the secondary metabolites such as alkaloids, phenols, and tannins to determine the anti-proliferative effect by inducing apoptosis on selected cancer cells A549 lung cancer, MCF-7 and MDAMB-231 breast cancer cells HeLa cervical cancer and HCT-116 colorectal cancer cells. The results have proved that the synergistic effect of the crude extract was potential in the inhibition of cancer cell proliferation than the secondary metabolites alone by regulating the biomarkers which are involved in development of cancer through enhancing the apoptosis of a cell. However, between the secondary metabolites the tannin extract has shown substantial inhibition of proliferation of breast cancer cells than alkaloids against colorectal cancer cells.

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Publications

1. **Srinivasa Krishnappa,** Yalpi Karthik and Manjula Shantaram*. Augmentation of *in vitro* cytotoxic potentiality of crude methanolic leaf extract of *Olea dioica* as a pivotal anticancer source. Biomedicine**.** 2023; 43(Suppl. 1):79-86. (Q4)

2. **Srinivasa Krishnappa,** Yalpi Karthik, Pratap G. K. and Manjula Shantaram*. Exploration of bioactive compounds from *Olea dioica* in Western Ghats of Karnataka using GC-MS**. 3Biotek. Accepted.**

Presentations

1. Presented a Poster on "Screening of Phytochemical And Antioxidant Activity Of Leaf Extracts From Two Important Medicinal Plants Of Western Ghat Region" during the national conference on "Biomedical Research And Innovations To Enhance Quality Of Life" under the auspices of 40th Annual Summit of Indian Association of Biomedical Scientists (IABMS) held from 31st October 2019 to 2nd November 2019 at Chettinad Hospital & Research Institute Kelambakkam, Kanchipuram Dist., Tamil Nadu.
2. Oral presentation on "Exploration of bioactive compounds from *Olea dioica* in Western Ghats of Karnataka using GC-MS for their antimicrobial and In-vitro cytotoxic activity" was presented in the international conference on "Recent Advances in Chemistry" held on 23rd November 2022, Organized by Dept. of Chemistry, Field Marshal K. M. Cariappa College, Madikeri.

Workshops and conferences attended

- Participated in the Interactive Workshop on "Areca nut and Human Health" held during 24th -25th July 2018 at Central Plantation Crops Research Institute, Kasaragod, Kerala.

- Participated in workshop on "Basics of cell culture and application in drug discovery" during the International Conference on "Current concepts on the role of Indian medicine and phytoceuticals in maintenance of health" under the auspices of 39th Annual Summit of Indian Association of Biomedical Scientists (IABMS) held from 15-17th November 2018 at Jnana Kaveri Post Graduate Centre of Mangalore University Chikka Aluvara, Kodagu.

- Participated in workshop on “DNA Damage Studies" at International Conference on Radiation Biology under the auspices of Indian Society of Radiation Biology (ICRB) theme being “Advances in Radiation Biology-Radiotherapy and Radiation Countermeasures” held on 4th October 2018 at K S Hegde Medical Academy, Deralakatte, Mangaluru.

- Participated in ten days workshop on "Plant Microbial Interaction for the production of therapeutic Compounds" held from 17th October 2019 to 26th October 2019 at Jnana Sahyadri, Kuvempu University, Shankaraghatta, Shimoga.

- Participated in 7-days training program on "Phytomedicines: Extraction, Purification, In vitro and In vivo studies" held from 24th July 2022 to 30th July 2022 Department of Biochemistry, Kuvempu University, Department of Biotechnology, Sahyadri Science College and JSS Academy of Higher Education and Research, Mysuru In association with Department of Science and Technology (DST-STUTI), Government of India

Lectures attended

1. Participated in Two Day Lecture Series in Molecular Genetics held on 24th and 25th August 2018 at K S Hegde Medical Academy, Deralakatte, Mangaluru in association with Karnataka Science and Technology Academy (KSTA)

Prof. Manjula Shantaram (Research Supervisor)

Mr. Srinivasa K (Research Scholar)

Date: **06-02-2024**

Place: **Chikka Aluvara**